Improved vessel preservation after 4 days of cold storage: Experimental study in rat arteries

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Background: **Cold storage of arteries for reconstructive and bypass surgery may result in injury of endothelial cells which may promote low perfusion and graft vasculopathy.**

Methods: **A recently developed *N*-acetyl histidine-buffered, potassium-chloride enriched, and amino acid-fortified vascular storage solution augmented with iron chelators deferoxamine (100 μmol/L) and LK 614 (20 μmol/L) was studied in the rat superior mesenteric artery and aorta with respect to: (1) potassium-induced vessel tone, (2) endothelium-dependent and -independent relaxation, and (3) endothelial nitric oxide synthase (eNOS) protein expression over 4-days cold storage (4°C).This solution was compared with traditional storage solutions, histidine-tryptophan-ketoglutarate (HTK) and physiological saline solution (PSS).**

Results: **Vessels stored for 4 days in the new solution were significantly better protected than those stored in traditional HTK or PSS. The protective effects comprised: (1) vessel tone development after stimulation with potassium-chloride solution, (2) endothelium-dependent and -independent vessel relaxation, and (3) eNOS expression. With iron chelators (deferoxamine 100 μM, LK 614 20 μM) present in the storage solution, endothelium-dependent relaxations (eNOS-dependent and K_{Ca}-channel-dependent) were fully maintained after 96 hours of cold storage. Endothelial cell structure was significantly better maintained after 96 hours in the new solution than in HTK or PSS solutions. Already, 2 hours of cold storage in HTK resulted in a significant loss of structurally intact endothelium. The structural changes correlated significantly with the diminished vessel relaxation capacity. Furthermore, tissue reductive capacity was only preserved after 96 hours storage if the new solution was used.**

Conclusion: **The new storage solution is superior to traditional HTK and PSS cold storage with respect to: (1) preservation of vessel structure and function; (2) the presence of iron chelators significantly improved protection of endothelial function; and (3) the new solution permits cold vessel storage for a minimum of 4 days with full maintenance of endothelial function and its coupling to smooth muscle. (J Vasc Surg 2009;50:397-406.)**

Vascular allografts are an important therapeutic option for surgical management of infrarenal aortic prosthetic graft infection.[1] For smaller vessels (<6 mm diameter), synthetic grafts have proved to be unsatisfactory and the attention is therefore directed toward the use of allografts.[2] Freshly isolated vessels and cryopreserved grafts have been used with similar clinical outcomes.[1,3-6] Despite their frequent application, current storage protocols need further improvement, in particular with respect to maintenance of endothelial cell function,[7-9] because impaired endothelial function may result in low perfusion after revascularization and promote postoperative graft vasculopathy.[8,10]

Cold storage (0-4°C) decreases tissue metabolism, and thus permits a better maintenance of cell energy homeostasis by anaerobic metabolism than warm storage. However, cold storage and subsequent re-warming may also activate specific injury processes, eg, production of reactive oxygen species triggered by a cold-induced rise of the cellular chelatable iron[11,12] which may deteriorate endothelial function.[13,14] A 24-48 hour period of successful endothelium and microvessel preservation during cold storage has been reported.[15-17] It seems highly desirable to extend this period and improve the availability of donor tissues. A recent experimental study reported substantial progress with respect to long-term storage of pig aortic segments over 3 weeks.[18] Based on the analysis of endothelial mitochondrial membrane potential and adhesion of platelets to endothelial cells, grafts were successfully stored in an *N*-acetyl histidine-buffered, potassium-chloride enriched, and amino acid-fortified storage solution augmented with iron chelators. This formula may help to avoid toxic side effects of histidine[19] and simultaneously utilize the cytoprotective effects of amino acids, eg, glycine and alanine in prevention of hypoxic injury.[18] The iron chelators may protect against iron catalyzed Fenton-type reactions during cold storage.[12]

The specific question addressed in this study was whether the previously developed new storage solution (solution 8 augmented with deferoxamine and LK 614)[18] permits a prolonged storage of arteries with full maintenance of endothelium-dependent vessel relaxation and endothelial nitric oxide synthase (eNOS) expression which is crucial for vessel function after vessel reconstructive and bypass surgery. The results clearly show that this is feasible.

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Table I. Composition of solutions used for storage of mesenteric arteries

	Solution 8	*Solution 9*	*HTK*	*PSS*
α-Ketoglutarate	2	2	1	—
Aspartate	5	5	—	—
Histidine	—	—	198	—
N-Acetylhistidine	30	30	—	—
Glycine	10	10	—	—
Alanine	5	5	—	—
Tryptophan	2	2	2	—
Sucrose	20	20	—	—
Glucose	10	10	—	5.5
Mannitol	—	—	30	—
Cl^-	103.1	103.1	50	128.7
$H_2PO_4^-$	1	1	—	1.18
SO_4^{2-}	—	—	—	1.17
HCO_3^-	—	—	—	25
Na^+	16	104	15	144
K^+	93	5	10	5.9
Mg^{2+}	8	8	4	1.17
Ca^{2+}	0.05	0.05	0.015	2.5
EDTA	—	—	—	0.027
pH	7.0	7.0	7.2	N.D.
Osmolarity	305.15	305.15	310	320

Concentrations are given in mmol/L, osmolarity is given in mosm/L.
HTK, Histidine-tryptophan-ketoglutarate; *PSS,* physiological saline solution.

METHODS

The experimental project conforming to National Institutes of Health (NIH) guidelines was approved by the German local authorities (permission 24D-9168.24-1-2006-23). Vessels isolated from male Wistar rats (Charles River Laboratories, Sulzfeld, Germany) anesthetized with urethane (1 mL/100 g i.p.) were flushed with physiological saline solution (PSS), histidine-tryptophan-ketoglutarate (HTK) solution, or the newly developed preservation solution (solution 8, Table I).[18] To explore potentially detrimental effects of a high potassium concentration,[20] we also tested in a modified solution with the potassium concentration reduced and the sodium increased (solution 9, Table I).[18] In a subset of experiments, solution 8 and HTK were augmented with the iron chelators deferoxamine (100 μmol/L) and LK 614 (20 or 10 μmol/L). After clearing off surrounding adipose tissue, the vessels were stored in the identical solution (4°C) for up to 96 hours. Then vessel segments were transferred to PSS solution and subjected to function testing 30 minutes after warming to 37°C.

Vessel function analysis. Mesenteric vessel function was measured with a Mulvany myograph (Power Lab/400, AD-Instruments, Spechbach, Germany), connected to a personal computer (PC) by interface Model 410A (Danish Myo Technologies, Aarhus, Denmark). Chart v4.1.2 software (AD Instruments, Spechbach, Germany) permitted data acquisition. Arteries (outer diameter 200-260 μm, cut 2 mm in width) were mounted to a PC-linked force transducer located in an organ chamber filled with PSS (equilibrated with 5/95% CO_2/O_2, pH 7.4, 37°C). Vessel rings were stretched with a resting tension equivalent to an intraluminal pressure of 100 mm Hg. Maximum contraction was induced with potassium enriched solution (KPSS), containing (mmol/L) 123.7 KCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 25 $NaHCO_3$, 5.5 glucose, 0.027 EDTA and 2.5 $CaCl_2$. After preconstriction with 7.5 μmol/L norepinephrine endothelium-dependent vasorelaxation was determined using acetylcholine (10^{-9}-$10^{-5.5}$ M) and endothelium-independent relaxation using sodium-nitroprusside (10^{-9}-10^{-4} M). Endothelial nitric oxide production was inhibited using L-N-monomethyl-arginine (L-NMMA, 100 μmol/L), cyclooxygenase with indomethacin (3 μmol/L) or diclofenac (10 μmol/L), Ca-dependent potassium channels (K_{Ca}) with charybdotoxin (50 nmol/L; large conductance K_{Ca}) and apamin (50 nmol/L; intermediate conductance K_{Ca}).

Protein expression. Vessels stored for 2 and 96 hours in either solution 8 augmented with deferoxamine (100 μmol/L) and LK 614 (20 μmol/L), HTK, or PSS solution (at 4°C) were studied without re-warming before protein isolation by chloroform/methanol precipitation. Protein content was determined by the amidoblack-method.[21] For Western blotting, 2.5 μg of total protein was used for each lane, separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amersham, Munich, Germany) by electro blotting. Protein expressions were assessed using antibodies against eNOS (BD Pharmingen, Heidelberg, Germany), β actin, and tubulin (Sigma, Taufkirchen, Germany). A modified custom-made ECL (Hybond ECL nitrocellulose membrane; Amersham Life Science, Arlington Heights, Ill) Western blotting detection method was used. Horseradish peroxidase anti-rabbit IgG goat anti-mouse IgG was obtained from Dianova (Hamburg, Germany). Blots were quantified using Image Gauge on an LAS 3000 analyzer (Fujifilm Life Science, Stamford, Conn).

Tissue reductive capacity. Rat aorta (n = 6) was stored in the different solutions for 96 hours and then gently warmed to 37°C within 1 hour. Thirty minutes later, resazurin (Alamar Blue) was added at a final concentration of 40 μM. The reduction of resazurin was assessed, as described before,[19] to measure reductive capacity which mainly reflects mitochondrial integrity and metabolism.

Lactate dehydrogenase release. Extracellular, ie, released lactate dehydrogenase (LDH) activity was measured in aliquots of the storage solution of rat aorta (n = 6) using standard procedures (Department of Clinical Chemistry, University Hospital Dresden). The LDH activity normalized to mg protein was expressed in $\mu mol\ s^{-1}\ l^{-1}$.

Endothelial structure. Rat mesenteric vessels stored for 2 and 96 hours at 4°C were studied. At the end of the storage period, the vessels underwent immediate fixation with glutaraldehyde (25%) and sodium cacodylate buffer (0.1 M) at a ratio 1:9 (vol/vol) without re-warming. After being washed in 0.1 M sodium cacodylate buffer (2 hours, 4°C), washed again, dehydrated in ethanol, and embedded in Epon 812, polymerization was carried out at 60°C. Specimens were viewed at 80 kV using an EM 906 microscope (Zeiss, Oberkochen, Germany). For semiquantitative

Table II. Scoring of endothelial cell injury

Score	*Description*
I	Well preserved endothelium
II	Intact endothelial layer with vacuole formation and edema
III	Endothelium detached in large fractions
IV	Complete denudation of the endothelium with single remaining cells

For illustration of injury please refer to Fig 7.

scoring, toluidine blue stained semi-thin sections (2-3 different sections per vessel) were used and estimated under a conventional light microscope (Olympus BX60; Olympus, Hamburg, Germany) with the investigator blinded to the experimental protocol. The scoring scheme is summarized in Table II.

Data analysis. Dose-response curves of relaxations were quantified by fitting the sigmoidal dose-response function $R(x)$ to the experimental data

$$R(x) = R_{\max} / 1 + \exp(- x + EC_{50} / \Delta R),$$

where x represents the logarithmic value of the applied dose. The resulting fit parameters R_{max}, EC_{50}, and ΔR were obtained with Bayesian data analysis and characterize each group of vessels. R_{max} reflects the maximum relaxation value and EC_{50} gives the concentration where half maximal relaxation is reached. The parameter ΔR specifies the slope of the transition around EC_{50}.

Statistics. Data are presented as mean values and their SEMs. Normal distribution was tested using the Kolmogorov-Smirnov test with modification after the Lilliefors test. Homogeneity of variances was assessed according to the Levene test. Differences of failure rates (vessels not responding to potassium stimulus) between groups were assessed using χ^2-statistics. Inter-group differences to stimuli were tested using one-way analysis of variance with post-hoc multiple comparisons after the Bonferroni correction. Regression analysis was performed according to the Spearman rank correlation. A P-value of .05 or less (two-sided) was taken to indicate a significant difference. Data analysis was performed using the SPSS program v15.0 (SPSS Inc, Chicago, Ill).

RESULTS

Effects of cold storage on potassium-induced vessel tone. Within 2 hours of isolation (control measurements), all mesenteric vessels studied (n = 116) responded to the KPSS developing significant vessel tone between 4.4 and 5.7 mN/mm (Fig 1). There was no significant difference between storage protocols after 2 hours. However, after 96 hours of storage at 4°C, major inter-group differences were evident with respect to vessel tone generation in response to KPSS (Table III). Whereas after storage in solution 8 with and without iron chelators, less than 10% of the vessels failed (no tone development during KPSS), the fraction of failing vessels was significantly increased after storage in HTK solution with and without iron chelators (63% and 38%). Storage in solution 9 gave similar results as storage in HTK solution. Thus, potassium-enriched solution (solution 8) protected better than storage at a low-potassium concentration (solution 9, HTK, Table I). All vessels stored in PSS solution for 96 hours failed to develop tone upon stimulation with KPSS. The superior preservation of vessels stored in solution 8 was highly significant vs the other storage solutions (Table III).

After 96 hours of cold storage in HTK, KPSS-stimulated wall tension was reduced as compared with 96 hours of storage in solution 8 (Fig 1). Addition of iron chelators did not significantly affect tension development in response to KPSS. Tension development after exposure to norepinephrine was generally 90-110% of that measured in the presence of KPSS.

Effects of different solutions and iron chelators on endothelium-dependent relaxation. We next explored the effects of solution 8 and of iron chelators on endothelium-dependent (acetylcholine, [ACH]) and endothelium-independent (sodium nitroprusside [SNP]) relaxations. Vessel relaxation in response to ACH was well preserved after 2 hours of cold storage in solution 8. However, without addition of iron chelators, ACH-evoked relaxation was significantly reduced after 96 hours of storage in solution 8 (from 92 ± 1 to 67 ± 10%, $P < .021$). This effect was prevented by addition of iron chelators. After 2 hours of storage in solution 9, vessel relaxation by ACH was reduced (76 ± 9%, $P = .028$ least-significant difference [LSD]-test). No significant differences resulted for SNP-induced relaxations with respect to storage solutions 8 vs 9 or with respect to the presence of iron chelators (2 and 96 hours).

Full concentration-response curves for ACH and SNP are shown in Fig 2 for vessels stored over 2 and 96 hours in either solution 8 (without and with augmentation with deferoxamine and LK 614), HTK, or PSS. This figure contains only data from vessels that developed tension after exposure to high potassium-chloride, ie, when failing vessel segments (Table III) were excluded from analysis. Then, concentration-response curves did not differ significantly from each other. However, if all vessels studied in the different storage groups were included in the analysis, then maximal relaxation toward ACH was significantly blunted in vessels stored 96 hours in cold HTK (Fig 3). The fact that function of vessels stored in HTK fell into two groups (preservation failures and successes) probably reflects the time course of the development of failure in this group (around 96 hours with some biologic variability). All vessels stored for 96 hours in PSS neither exhibited vessel tone development when exposed to KPSS or norepinephrine nor did the vessels show any relaxation toward ACH or SNP ($P < .0001$ vs all other groups). In vessel segments stored 48 hours in PSS, the endothelium-dependent relaxation was already abolished >90%, whereas direct smooth muscle relaxation was still present at this time point (data not shown).

Maintenance of NO- and EDHF-mediated responses. To quantitatively assess the potential of endothelium-dependent relaxation in vessels stored in solution 8 aug-

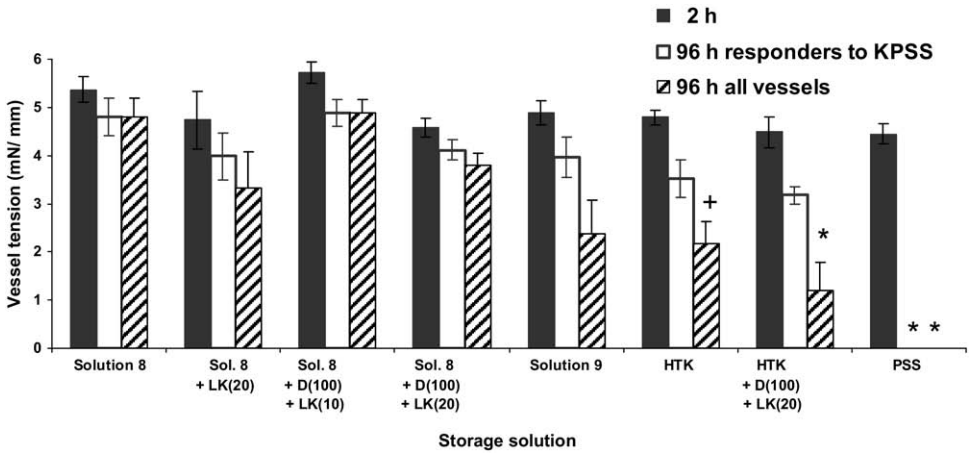

Fig 1. Vessel tension development after stimulation with high potassium-chloride solution (123.7 mM). Results are shown for vessels stored for 2 (*black bars*) and 96 hours (*open and hatched bars*). *Open bars* represent vessels which responded to potassium exposure (potassium enriched solution [KPSS]) with tone development (Table III), *hatched bars* represent all vessels studied. The variations in solution 8 and histidine-tryptophan-ketoglutarate (HTK) designate differences in the addition of iron chelators LK 614 (LK) or deferoxamine (D). Numbers in parenthesis indicate concentrations in μM. $^{+}P < .001$, $*P < .0001$ vs the respective condition using solution 8. *PSS,* Physiological saline solution; *h,* hours.

Table III. Functioning vessels after 2 and 96 hours storage in different solutions

	Functioning vessels after 96h/2h	*Significance vs*			
		Sol. 8	*Sol. 8 + LK(20)*	*Sol. 8 + LK(10) + D(100)*	*Sol. 8 + LK(20) + D(100)*
Solution 8	8/8	—	N.S.	N.S.	N.S.
Solution 8 + LK(20)	5/6	N.S.	—	N.S.	N.S.
Solution 8 + D(100) + LK(10)	9/9	N.S.	N.S.	—	N.S.
Solution 8 + D(100) + LK(20)	30/33	N.S.	N.S.	N.S.	—
Solution 9	6/10	$P < .05$	N.S.	$P < .05$	$P < .05$
HTK	13/21	$P < .05$	N.S.	$P < .05$	$P < .01$
HTK + D(100) + LK(20)	3/8	$P < .01$	N.S.	$P < .01$	$P < .001$
PSS	0/21	$P < .001$	$P < .0001$	$P < .001$	$P < .001$

Test stimulus was KPSS. Functioning vessels are defined as specimen developing tone during exposure to KPSS. Numbers in parenthesis give concentrations of LK 614 (LK) and deferoxamine (D) in μmol/L. *P*-Values indicate significance levels for inter-group comparisons. Vessels stored in HTK and HTK + LK(20) + D(100) did not differ significantly. *N.S.,* Not significant; *h,* hours; *HTK,* histidine-tryptophan-ketoglutarate; *PSS,* physiological saline solution; *KPSS,* potassium enriched solution.

mented with deferoxamine and LK 614, the nitrous oxide (NO)-dependent and the endothelium-derived hyperpolarizing factor (EDHF)-dependent components of vessel relaxation were quantified (Fig 4). L-NMMA shifted the concentration-response curve of ACH significantly to the right. In presence of L-NMMA addition of apamin plus charybdotoxin completely blunted the effects of ACH. Blockers of cyclo-oxygenases (indomethacin n = 12 or diclofenac n = 8) did not have any significant effect on the relaxation toward ACH (data not shown). Blockage of K_{Ca}-channels by apamin plus charybdotoxin reduced the maximum of the concentration-response curve of SNP ($P < .05$). All effects were similar after 2 and 96 hours cold storage.

Effects on protein expression. Fig 5 depicts expression of eNOS, tubulin, and β-actin proteins in mesenteric artery given as the ratio of measurements after 2 and 96 hours cold storage. Whereas protein expression was well preserved if storage was done in solution 8 augmented with iron chelators or HTK, it was blunted after 96 hours storage in PSS (protein loss 70% to 83%).

Effects on endothelial cell structure. Fig 6 depicts different degrees of endothelial damage as assessed by light and electron microscopy in mesenteric arteries. Criteria for damage scoring are given in Table II and illustrated in Fig 7. Endothelial cell structure was well maintained after 2 hours of cold storage in solution 8, augmented with deferoxamine and LK 614, or PSS. However, injury was evident in vessels stored in traditional HTK solution even after 2 hours ($P = .006$). After 96 hours of storage in PSS, endothelial cell structure was severely deteriorated ($P = .008$), whereas endothelial structure was preserved in ves-

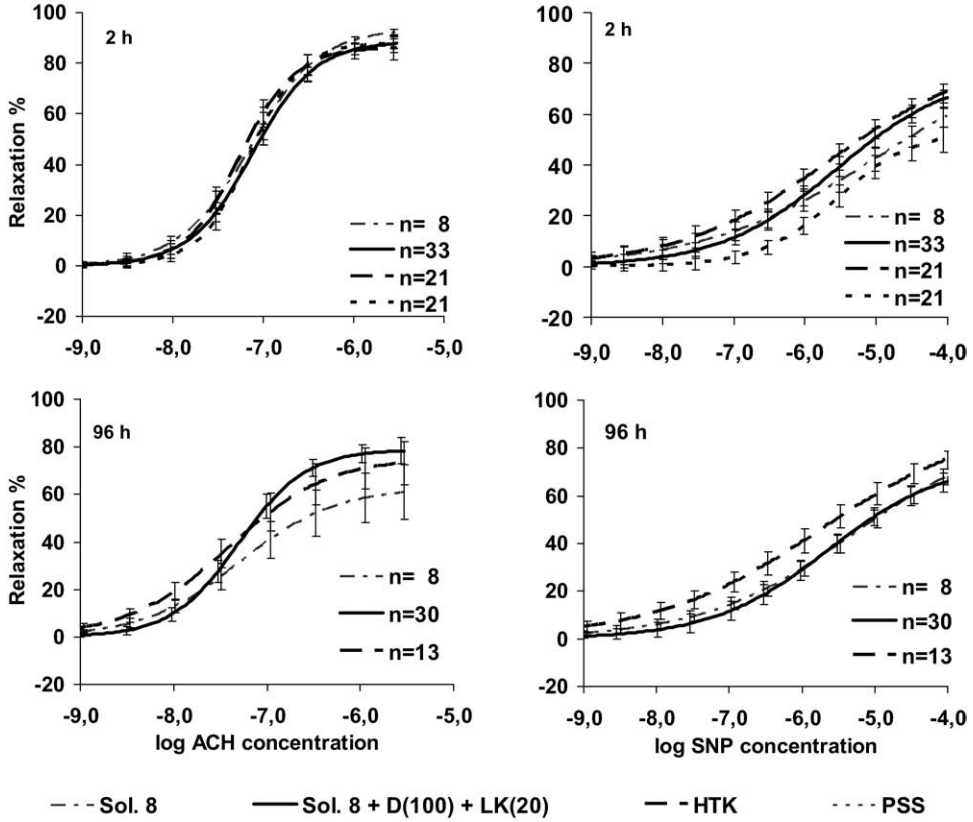

Fig 2. Relaxation properties of vessel segments responding to the potassium enriched solution (KPSS) stimulus with tone development (note that functioning vessels differed between groups, Table III). Shown are relaxations to stimulation with acetylcholine (ACH) and Na-nitroprusside (sodium nitroprusside [SNP]) after 2 hours **(upper panel)** and 96 hours **(lower panel)** storage. Storage conditions were solution 8 without and with deferoxamine (100 μM) plus LK 614 (20 μM), respectively, histidine-tryptophan-ketoglutarate (HTK), or physiological saline solution (PSS). Curves as fit to the function *R(x)* are shown. Experimental data is shown as mean ± SEM. Numbers indicate the number of vessels included. Data for all vessels studied are shown in Fig 3.

sels stored in solution 8 augmented with iron chelators. After 96 hours of storage in HTK, endothelial cells exhibited a degree of damage similar to that after 2 hours of HTK storage. Endothelial injury score correlated negatively with acetylcholine induced vessel relaxation ($r = -0.36$, n = 36, $P = .03$). Thus, the structural changes may in part account for the diminished vessel relaxation.

Protective effects in rat aortic tissue. To assess whether the protective effects seen in mesenteric artery segments may be extrapolated to large conduit arteries, further measurements were performed in rat aorta. Expression of eNOS, tubulin, and β-actin after storage in different solutions was similar to that found in mesenteric arteries (results not shown). LDH activity released from aortic rings was similar for all storage solutions after 2 hours at 4°C (5.9 ± 2.2 μmol s^{-1} l^{-1} per mg protein). However, after 96 hours of cold storage released-LDH activity was 17.8 ± 5.2 ($P = 0.05$ vs. control), 13.9 ± 2.6 and 7.0 ± 1.3 μmol s^{-1} l^{-1} per mg protein, respectively, for HTK, PSS, and solution 8 augmented with iron chelators. Resazurin reduction was significantly higher ($P < .001$) after 96 hours storage in solution 8 augmented with iron chelators (108 ± 13%, ie, similar to unstored controls) than after storage in PSS (48 ± 13%) or HTK (41 ± 6 %). Already after 2 hours of cold storage in HTK, reductive capacity declined (Fig 8).

DISCUSSION

The major finding of the present study is that rat mesenteric arteries and aorta stored at 4°C in the new vascular preservation solution 8, augmented with iron chelators deferoxamine and LK 614, were significantly better protected with respect to function and structure than vessels stored in traditional HTK or PSS. The protective effects of this new solution were objectified by: (1) vessel tone development after stimulation with KPSS, (2) endothelium-dependent vessel relaxation, (3) endothelium-independent vessel relaxation, (4) maintenance of protein expression, (5) tissue reductive capacity, and (6) maintenance of endothelial cell structure. In contrast, vessels stored in HTK for only 2 hours exhibited a significant loss of structurally intact endothelium and tissue reductive capacity declined. Most notably, after 96 hours

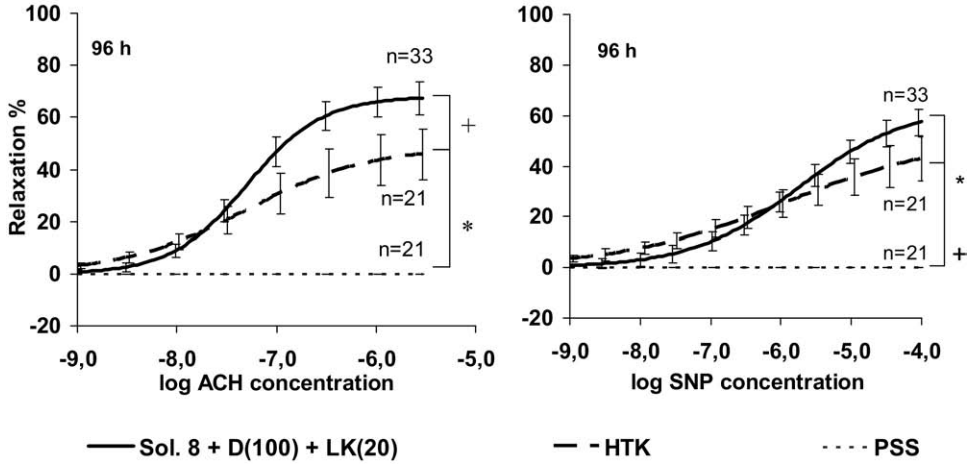

Fig 3. Relaxation properties of all vessel segments studied irrespective of tone development during potassium enriched solution (KPSS) stimulus (Table III). Shown are relaxations to stimulation with acetylcholine (ACH) and Na-nitroprusside (sodium nitroprusside [SNP]) after 96 hours storage. Number of experiments is indicated by the number next to respective curve. **Left panel:** $^{+}P < .004$ solution 8 augmented with iron chelators vs histidine-tryptophan-ketoglutarate (HTK), $*P < .0001$ physiological saline solution (PSS) vs other solutions. **Right panel:** $P < .0001$ PSS vs HTK (+) and PSS vs solution 8 augmented with iron chelators (*). *h*, Hours.

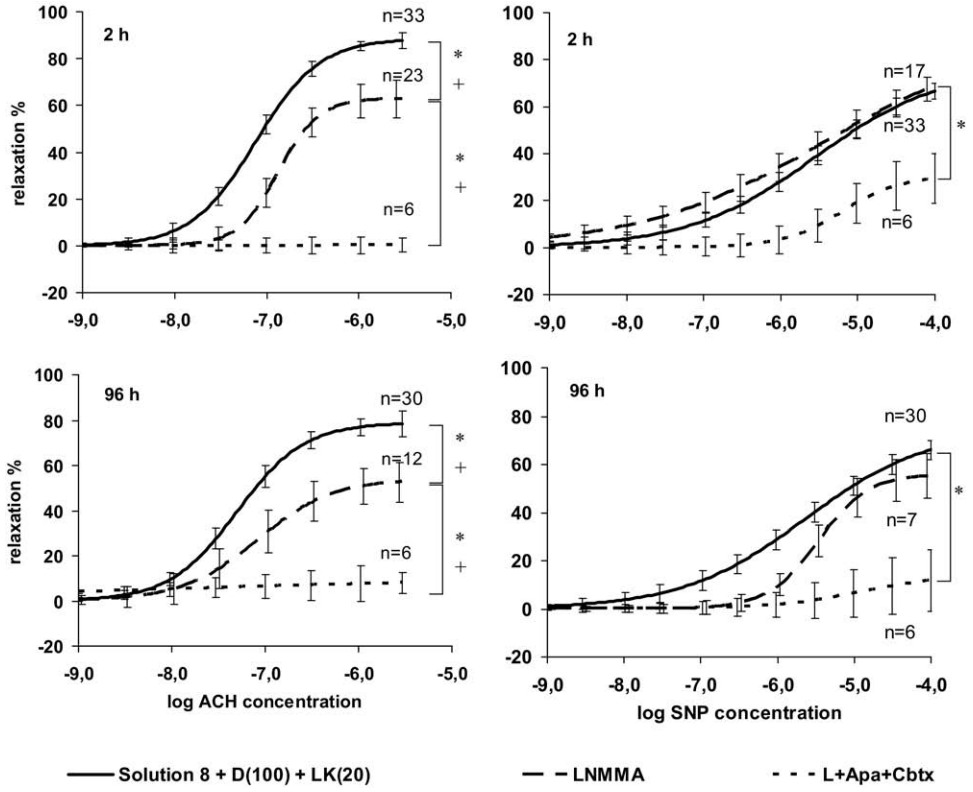

Fig 4. Vessel relaxation after stimulation with acetylcholine (ACH) and Na-nitroprusside (sodium nitroprusside [SNP]) after 2 hours **(upper panels)** and 96 hours **(lower panels)** storage in solution 8 augmented with deferoxamine (100 μM) plus LK 614 (20 μM). Concentration-effect curves are shown as fit to the function *R(x)* for control conditions (*continuous line*), after application of L-N-monomethyl-arginine (L-NMMA) (*broken line*), and after application of L-NMMA plus apamin plus charybdotoxin (*dotted line*). *Indicates a significant difference of maximal relaxation, $^{+}$indicates a significant difference of EC_{50}. Numbers indicate the number of vessels tested.

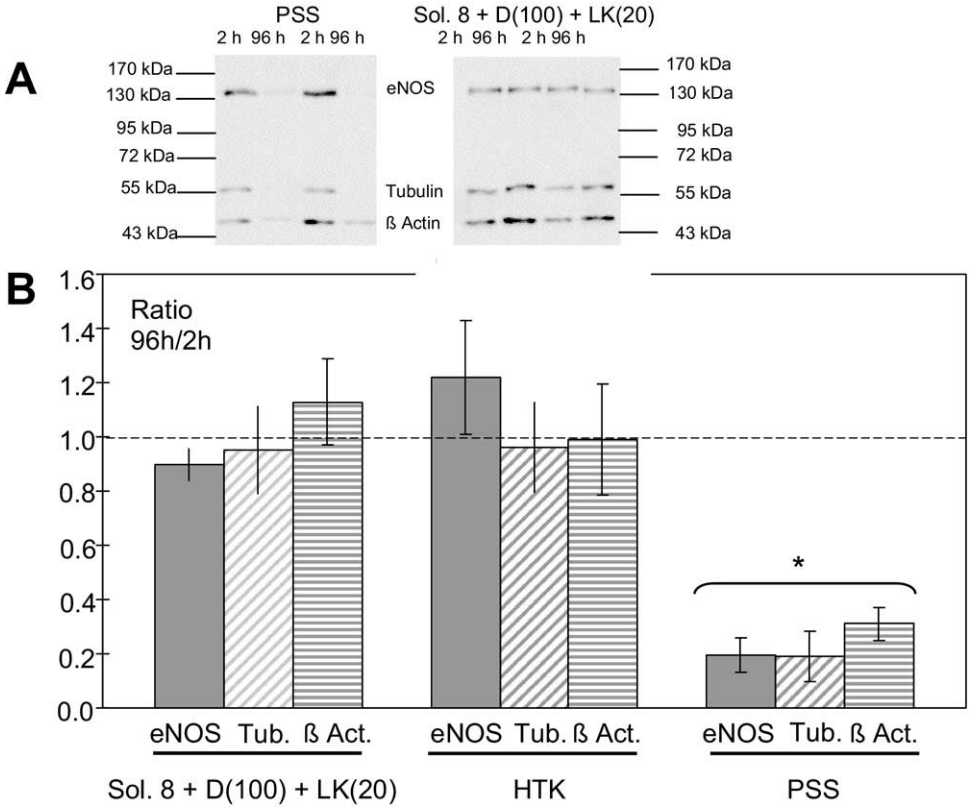

Fig 5. Protein expression of endothelial nitric oxide synthase (eNOS), tubulin and β-actin after 2 and 96 hours cold storage. Storage conditions were solution 8 augmented with deferoxamine (100 μM) plus LK 614 (20 μM), histidine-tryptophan-ketoglutarate (HTK) or physiological saline solution (PSS). Typical Western blots are shown under **(A)** for 2.5 mg total protein per lane. Mean values of 13 (solution 8), 10 (HTK), and 5 vessels (PSS) are shown under **(B)**. Data is expressed as the ratio of measurements performed after 2 and 96 hours. $*P < .01$ PSS vs other groups. *h*, Hours.

storage in traditional HTK or PSS severe functional and structural injuries were evident.

Differentiation of endothelium-dependent vessel relaxation. The contributions of NO and K_{Ca}-channels were assessed using validated pharmacological inhibitor protocols.[22,23] L-NMMA is a potent inhibitor of eNOS in the concentration used. Because block of cyclooxygenase by indomethacin and diclofenac did not significantly reduce the relaxation toward acetylcholine, the remaining relaxation of rat mesenteric artery in the presence of L-NMMA is most likely mediated via activation of K_{Ca}-channels.[24] This is supported by the complete elimination of acetylcholine-induced relaxation after combined block of intermediate and large (charybdotoxin) and small (apamin) conductance K_{Ca}-channels (Fig 4). In addition to smooth muscle, endothelial cells are also equipped with K_{Ca}-channels.[25] Functional involvement of endothelial K_{Ca}-channels in NO release has been demonstrated in rat mesenteric artery.[26] Thus, block of endothelial K_{Ca}-channels may have contributed to blunting the relaxation effect of acetylcholine. However, apamin and charybdotoxin also blocked smooth muscle K_{Ca}-channels (Fig 4) evidenced by the reduced relaxation after Na-nitroprusside stimulation. Vasorelaxation toward acetylcholine after eNOS inhibition (L-NMMA) and cyclooxygenase inhibition (which did not play a role in the model used) is interpreted as EDHF-mediated relaxation.[22,23]

The expression of eNOS as a marker enzyme of endothelium-dependent relaxation was reduced after extended storage in PSS in relation to other proteins (tubulin, β-actin) (Fig 5). In agreement with this result is the missing vessel tone development after KPSS exposure (Fig 1), the missing effects of acetylcholine and Na-nitroprusside on vessel tone (Fig 3), reduced reductive capacity (Fig 8) and the severe endothelial cell injury (Fig 6) after 48 hours of cold storage in PSS. This generalized vessel injury is preceded by an endothelium specific injury of vessel relaxation until 48 hours.

Vascular storage injury and chelatable iron. A recent study[15] has addressed endothelial structural changes of rat abdominal aorta after 24 hours of storage in University of Wisconsin (UW), Collins, and Euro-Collins solutions. Endothelial cell structure was maintained after 24

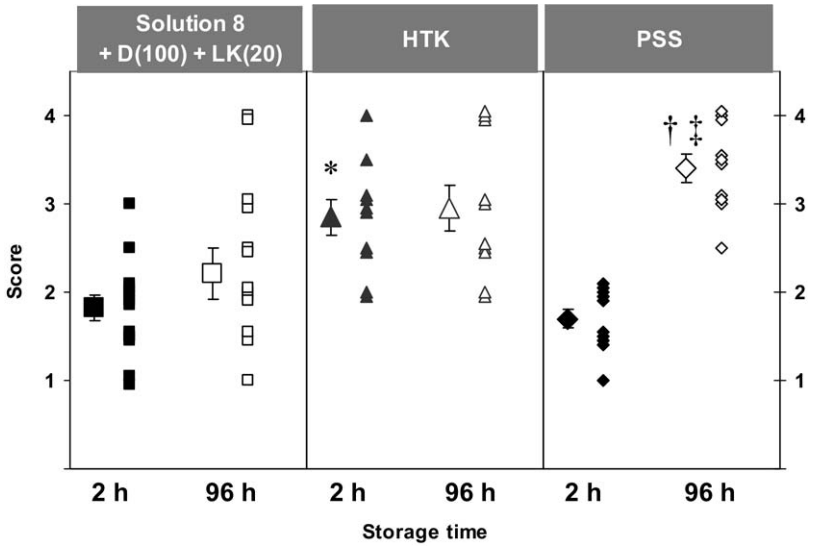

Fig 6. Endothelial injury scores after 2 and 96 hours cold storage. Storage conditions were those given in the legend to Fig 5. Scoring criteria are shown in Table II and exemplified in Fig 7. *Significantly different vs solution 8 augmented with iron chelators and physiological saline solution (PSS) at 2 hours, †significantly different vs PSS at 2 hours, ‡significantly different vs solution 8 at 96 hours. Vessels studied were 14 and 13 for 2 and 96 hours solution 8, respectively, and 10 for all other conditions. *h*, Hours.

hours only in UW-solution. In the present study, HTK storage resulted in structural endothelial cell injury early during cold exposure (Fig 6). However, when re-warmed after 2 hours of cold exposure in HTK, vessel relaxation toward acetylcholine and Na-nitroprusside was still present in a significant fraction of the vessels (Figs 2 and 3) if KPSS-induced vessel tone development was maintained (Table III). A loss of rat coronary resistance vessel dilatory capacity was also evident after 8 hours of cold storage in HTK solution.[14] This impairment was ameliorated by augmenting HTK with iron chelators. Similarly, the endothelial cell injury in cold-stored porcine aorta was inhibited by the iron chelators deferoxamine and LK 614,[18] and in the present study the protective effect of solution 8 is augmented by the presence of the same iron chelators. This confirms in vitro data (cultured endothelial cells) that chelatable iron is an important factor causally involved in cold-induced endothelial injury[11,27-29] and adds further evidence that this concept is valid for clinically-relevant storage conditions.

Protective principles of the new vascular preservation solution. Part of the protection by the new vascular preservation solution is provided by the iron chelators. Deferoxamine is a strong hexadentate iron chelator that has been in clinical use for the treatment of hemochromatosis for decades. Because it has the disadvantage of poor membrane-permeability, it is supplemented with the new chelator LK 614, a small aromatic hydroxamic acid derivative, that is supposed to provide the primary protection against the adverse effects of intracellular chelatable iron during cold preservation.[18]

Already in the absence of iron chelators, solution 8 provided superior protection when compared to HTK and PSS. Solutions 8 and 9 are *N*-acetyl histidine-buffered; this buffer has the advantage over histidine (the buffer of HTK solution) that it does not share the toxic side effects of histidine[19] and over bicarbonate (the buffer in PSS) that its p*K* value of 7.2 (at room temperature) is better suited to prevent (severe) acidosis under hypothermic conditions. Also, it does not require the balance with CO_2. In addition, 10 mM glucose provide substrate for the highly glycolytic endothelial cells[30,31] and cytoprotective amino acids, especially glycine and alanine, provide protection against hypoxic injury.[32-34] Furthermore, the moderately acidic pH has proven advantageous in porcine aortic segments.[18] It was beyond the scope of the current study to evaluate these single components individually.

Potassium chloride-enriched solution 8 offers solid protection of arterial vessels during extended periods of cold storage (Figs 1-6, Fig 8, Table III). Solution 9, which differs from solution 8 only by the concentrations of potassium and sodium, did not protect as well. This is in agreement with results recently found in pig aorta,[18] and it is remarkable because potassium-rich solutions are widely regarded as toxic for the endothelium (for a general discussion on this point see Wille et al[18]). In particular, it has previously been concluded that high potassium storage solutions may be detrimental for EDHF-mediated vasorelaxation in pulmonary microvessels[20] and coronary vessel preparations.[35,36] Our results with apamin and charybdotoxin argue against a generalization of this notion and suggest that EDHF-mediated relaxation is well preserved in the new solution 8.

Study limitations. As an experimental model we have chosen the rat aorta and mesenteric artery, because these vessels have been well characterized. In particular, the

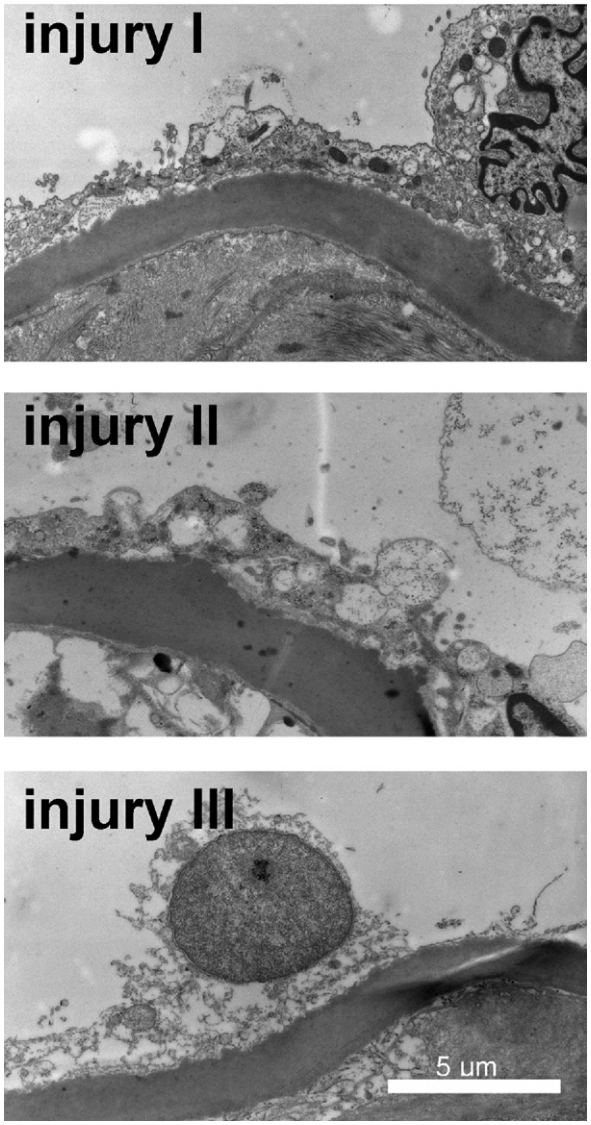

Fig 7. Micrographs illustrating the injury scores. Injury grade IV resulted in a complete loss of endothelium (not shown).

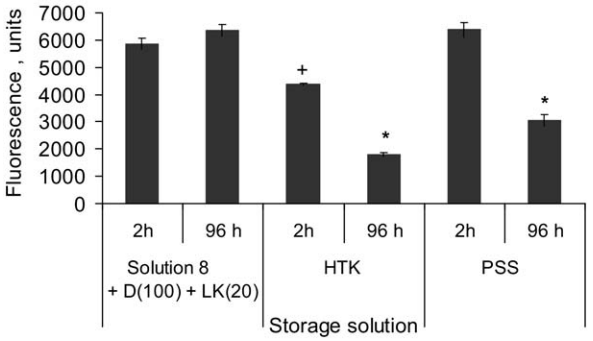

Fig 8. Reduction of Alamar Blue (resazurin) after 2 and 96 hours of cold storage of rat aortic segments in solution 8 augmented with iron chelators (solution 8 + D[100] + LK[20]), histidine-tryptophan-ketoglutarate (HTK) and physiological saline solution (PSS). Reduction assays were performed after warming samples to 37°C. $^{+}P = .07$, $^{*}P < .0001$ vs solution 8 augmented with iron chelators (96 hours), n = 6 for each bar. *h,* Hours.

mechanisms of endothelium-dependent and -independent relaxation have been studied in detail in rat mesenteric artery,[26,37] which shows similar mechanisms of relaxation as the human vessel.[38] Furthermore, vessels taken from standard laboratory animals can be studied in a well standardized manner, unaffected by pathologic processes as they may occur in tissues obtained from humans. Nonetheless, future studies using human vessels will be required to extend the promising results of the present study to the clinical arena.

Future potential of improved cold storage. Because of appreciable clinical importance, the benefits and disadvantages of specific storage protocols need to be studied in more detail and be comprehensively documented. Current clinical experience has resulted in various vessel storage protocols, which may rather reflect experiences of different surgical teams than systematic research. Systematic research, however, is required to improve protocols for extended vessel storage periods. This will permit to: (1) extend storage protocols for banking of grafts for later transplantation, (2) separate the time of vessel explantation and bypass surgery, (3) store vessel segments for later revision surgery, and (4) improve functional and structural quality of the graft.

In conclusion, the present study provides compelling evidence that the *N*-acetyl histidine-buffered, potassium chloride-enriched, amino acid fortified storage solution 8 allows superior preservation of arteries over several days of cold storage. Augmentation with the iron chelators deferoxamine and LK 614 fully maintains the endothelium-dependent vessel function over at least 4 days.

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AUTHOR CONTRIBUTIONS

Conception and design: AD, UR
Analysis and interpretation: BZ, PD, AD
Data collection: BZ, BM, MK
Writing the article: AD, UR
Critical revision of the article: AD, UR, PD, MK
Final approval of the article: BZ, PD, BM, MK, UR, AD
Statistical analysis: BZ, PD
Obtained funding: AD
Overall responsibility: AD

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